AD_____

GRANT NUMBER DAMD17-97-1-7277

TITLE: Identification of Surrogate Markers for Estrogen Receptor Action in Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Julianne M. Galluzzo

CONTRACTING ORGANIZATION: Duke University
Durham, North Carolina 27710

REPORT DATE: July 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1998	Annual (1 Jul 97 - 30 Jun 98)

4. TITLE AND SUBTITLE
Identification of Surrogate Markers for Estrogen Receptor Action in Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-97-1-7277

6. AUTHOR(S)
Galluzzo, Julianne M.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Duke University
Durham, North Carolina 27710

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The human estrogen receptor (ER) is a ligand-inducible transcription factor that mediates the biological effects of the steroid hormone estrogen. Different ER ligands can regulate the expression of specific subsets of genes. While "pure" antiestrogens like ICI antagonize ER activity in all contexts, other antiestrogens such as tamoxifen can act as partial agonists in some situations and antagonists in others. Recently a second estrogen receptor (ERb) has been identified, and I have shown that this receptor is less sensitive to estrogen and antiestrogens. While both receptors are expressed in human breast tumors, I have been interested in identifying novel ER genomic targets, and studying their differential regulation by both ERs during the administration of agonists and antagonists.

Using differential display PCR, I identified the hMIP gene (human mitochondrial intermediate peptidase) based on its upregulation by both ER agonists and antagonists. My current studies are aimed at further characterizing the ER regulation of hMIP. Furthermore, I have expanded my studies to include a dissection of the mechanism by which ERa and ERb show differential sensitivities to hormones so that we can better understand the regulation of our novel ER-regulated genes in the breast.

14. SUBJECT TERMS
Breast Cancer, tamoxifen, antiestrogen, estrogen receptor, gene regulation

15. NUMBER OF PAGES
20

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

JMG For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

JMG In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

JMG In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

JMG In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Julie M. Gallegos 7/15/98

PI - Signature Date

TABLE of CONTENTS

INTRODUCTION

Breast cancer is the leading cancer among women, and its associated mortality rates have not changed significantly over the past two decades (1). Evaluation of primary tumors has shown that 60% express the estrogen receptor (ER), which confers estrogen responsiveness to the tissue (2). In contrast, ER is poorly expressed in normal mammary epithelial cells. While the role of the receptor is not clear, estrogen exhibits mitogenic effects in ER positive breast cancer cells. This action is likely due to the modulation of expression of key regulators of growth, such as transforming growth factor-β (TGF-β), a direct target of ER. Therefore, for the past eighteen years antiestrogens have been used to block the proliferative effects of estrogen in breast cancer patients and halt disease progression (3). While tamoxifen is one of the mainstay therapies for the treatment of hormone-dependent breast cancer, most women develop resistance to treatment within 5 years (4). Many models of tamoxifen resistance have been proposed, which include estrogen receptor mutations, change in the levels of paracrine growth factors and the modulation of downstream events in ER pathways (5, 6). However, it has become clear that we need to first define the targets and mechanisms of estrogen and tamoxifen action in order to understand how cell growth processes escape from endocrine control during antiestrogen therapy.

The ER is a member of the nuclear receptor superfamily (7), whose members include the receptors for progesterone (PR), glucocorticoids (GR), androgens (AR) and aldosterone (MR). The receptors are ligand inducible transcription factors that share a common mechanism of action. In the absence of hormone, the receptors reside in the cytoplasm as part of a multiprotein complex that includes heat-shock protein 90 (hsp90), hsp70, p59 and other proteins (8). Ligand binding induces conformational changes within the receptors, which results in displacement of the associated proteins, receptor dimerization and nuclear localization. The receptor homodimers then bind with high affinity to specific recognition sequences (steroid response elements, or SREs) which are located within the regulatory regions of target genes. From these sequences steroid receptors can interact with the general transcription apparatus (GTA) in order to modulate the rate of transcription of their targets.

The ability of ER to modulate transcription is dependent upon the cellular and promoter context (9). The transactivating function of ER is mediated by two distinct domains, an amino terminus Activation Function-1 (AF1) and a carboxyl terminus located AF2. While on a minimal promoter both the activity of both AFs are required, our laboratory and others have shown that AF1 and AF2 can act independently on complex

promoters (9, 10). The activity of AFs in certain contexts determines which genes are transcribed, and this contributes to the cell specificity of some ER actions.

The ability of ER to regulate transcription is also dependent upon the ligand. Our lab has shown using in vitro assays that ER agonists and antagonists induce distinct receptor conformations following interaction with different classes of ligands (11). We believe that this enables cells to distinguish between agonist and antagonist bound receptor. The pure ER antagonist ICI antagonizes ER function in all cell and promoter contexts. However, some antiestrogens such as the triphenylethylene derived compounds (tamoxifen, raloxifene, GW7604) can act as ER partial agonists in some cell and promoter contexts, and antagonists in others. The current model is that tamoxifen inhibits ER AF2 activity, and so its antagonistic effects are manifest in those contexts where AF2 activity is required. Conversely, tamoxifen's agonist properties are thought to be mediated through ER AF1 activity, and in those contexts where AF1 activity alone is required, tamoxifen can modulate transcription of ER target genes (9).

Two forms of the human estrogen receptor have been described, the widely characterized ERα, and the more recently discovered ERβ (12). The receptors have similar structures, and display high conservation in the central DNA-binding domain, moderate conservation of the ligand-binding domain in the C-terminus, but diverge considerably in the N-terminus. ERα and ERβ have been shown to bind the same ligands with similar affinities and to regulate transcription of the same promoters. The receptors are expressed in many regions (including the breast, bone, brain, and the cardiovascular, immune, reproductive and digestive systems) where ERα and ERβ have been shown to both colocalize and to display unique distributions (13, 14). When the two receptors are coexpressed they preferentially form ERα/ERβ heterodimers, that are capable of activating transcription from estrogen-responsive enhancers (15, 16). This suggests the existence of novel pathways of estrogen signaling, where any variations in the activity of one ER subtype could impact the activity of the other.

While several ER regulated genes have been previously described, the new advances in gene hunting technology have facilitated the very recent identification of additional genes with interesting paradigms of ER regulation. Greene et al. (17) have shown that the MCP-1 (monocyte chemoattractant protein 1) message is downregulated by estrogen through a mechanism in which an agonist-bound ER antagonizes NF-κB binding to the MCP-1 promoter. Furthermore, Montano and Katzenellenbogen (18) have recently described the ability of tamoxifen to upregulate the quionone reductase gene, and Silva et al. (20) have also observed the downregulation of CD36 by tamoxifen. However, since these antiestrogen regulated genes have not been shown to be direct targets of the ER, these

findings suggest that there are upstream events in the tamoxifen-ER pathway that need to be explored. Furthermore, these recent studies suggest that there are still a wealth of novel estrogen and antiestrogen regulated genes to be discovered. We feel that continuing to identifying these genes will contribute to the understanding of ER biology and hormone resistance in breast cancer. My particular interest is in identifying direct targets of ER so that I can study the mechanistic basis of their regulation at the promoter level.

I have been using differential display PCR to identify surrogate markers of estrogen and antiestrogen action in human breast cancer cells. One gene has been the recent focus of further study due to its unique ability to be upregulated by both ER agonists and antagonists. I have since cloned the full length cDNA and identified the gene as hMIP (human mitochondrial intermediate peptidase), an enzyme important in cellular respiration. My current studies are aimed at characterizing the ER regulation of hMIP and defining the promoter sequences important for its regulation by agonist and antagonist. I have also expanded our studies to include a pharmacological comparison of ERα and ERβ in response to estrogens and antiestrogens. I have found that ERα is much more sensitive to estrogen and antiestrogen administration than ERβ, which may be significant *in vivo*, since different breast tumors have been shown to express different amounts of the two ERs. These studies will enable me to use the novel ER regulated genes that I have been identifying to study the differential regulation by ERα and ERβ in response to estrogen and many of the pharmaceutically-derived antiestrogens.

BODY

1. Isolation of mRNA enriched for estrogen and tamoxifen regulated genes.

Supporting Data

I have been using MCF-7 cultured cells for my studies. This human breast cancer derived cell line demonstrates growth and gene expression when administered estrogen, in a capacity that mimics hormonal effects on ER positive tumors *in vivo* (20). Furthermore, MCF-7 cell derived tumors have been used to study hormone resistance in animal models (21, 22). Therefore, these models could be used in the future to define those genes which escape endocrine control when cells acquire resistance. Although the use of differential display PCR to study estrogen receptor activity is not novel, we feel that the specific approach that we are using will add significantly to these studies.

We and others have observed a significant ligand independent ER activity, which results in the expression of its target genes in the absence of hormone. This phenomenon has been attributed to activation of ER and other steroid receptors by signaling pathways induced by dopamine, growth factors and cAMP (23). Although several attempts have been made by altering cell media constituents, I've been unable to obtain low ER activity in absence of hormone. However, since the success of differential display is dependent upon distinct patterns of gene expression between hormone treatment groups, the technique has required that we have low basal levels of ER activity for comparative purposes between RNA populations from untreated and hormone treated cells. However, we have avoided this problem by using cells treated with the pure ER antagonist ICI as our "no hormone" group (Figure 2). This compound is a pure antagonist that prevents ER activation and subsequent transcription of its target genes (11). Consequently, ICI decreases the basal levels of ER activity by inhibiting both ligand dependent and independent receptor activity.

I have determined the time course for the induction of target genes of ER. This was accomplished by treating MCF-7 cells with the ER agonist 17B-estradiol for different lengths of time, and by monitoring the expression levels of two ER regulated genes, PS-2 and the PR by northern analyses. A maximum induction was observed at 24 hours, and this initially established our time point for the isolation of RNA following treatment with estrogen and tamoxifen. However, our concern with using long induction periods was that we might observe gene expression due to secondary effects of cell proliferation and also detect gene products further down in the cascade. This was apparent in my first differential display experiments where I identified genes that appeared to be regulated by the ER, but

were later determined to be artifacts of downstream proliferative pathways. For our most current studies, these possibilities were eliminated by the use of cycloheximide at a concentration known to inhibit greater than 95% of all protein synthesis (24). In addition, I have performed cell cycle analysis of MCF-7 cells treated with estrogen for various time points. I have determined that a 6-hour induction did not yet drive cell cycle progression (and proliferation), but was still sufficient for induction of ER regulated cDNAs. Therefore, we chose this as the hormone induction time for our most current studies.

Methods

MCF-7 cells were grown in RPMI supplemented with 10% FCS, and treated with 50μM cycloheximide and 10^{-7}M hormone (ICI, tamoxifen or estrogen). After 6 hours, total RNA was isolated by the guanidinium thiocyanate or Ultraspec RNA (Biotecx) method. Expression of PS-2 has been used as a positive control for the induction of ER regulated genes in the RNA population that has served as our differential display template. Cell cycle analysis was performed by treating MCF-7 cells with estrogen for various time points, staining with propidium iodide and study of the cell cycle distribution by facs analysis.

2. Identification of estrogen and tamoxifen regulated mRNAs using differential display PCR.

Supporting Data

The differential display technique was developed for the identification of differences in gene expression between distinct RNA populations (25). Its use permits comparisons of gene transcription between distinct cell types, cell developmental stages or as in our study, cells that have been administered different drug or hormone treatments. Total RNA is reverse transcribed to form cDNA, and the partial cDNA sequences are amplified using different sets of primers. This ensures that only a set of 50-100 mRNAs are amplified at once, which permits display of the PCR reactions on sequencing gels. The cDNAs for each group to be compared are run in adjacent lanes, and differences in the expression levels of specific genes can be visually identified. The advantage of this technique for our study is that it enables us to simultaneously compare the gene expression patterns of MCF-7 cells exposed to different hormone treatments (estrogen, tamoxifen, and an untreated group). This allows us to identify novel genes that are induced or downregulated following the administration of each compound, and those whose expression is regulated by both ER ligands.

I have performed differential display PCR in our laboratory using this protocol. Upon comparison of mRNA from cells treated with estrogen, tamoxifen and ICI, I had

identified 13 genes upregulated by estrogen and 1 by tamoxifen, and one that was downregulated by estrogen. These cDNAs were selected based upon their differential hormone regulation in duplicate PCR reactions.

Methods

RNA derived from MCF-7 cells treated with estrogen, tamoxifen, and ICI was obtained in aim #1. We have been using the GenHunter (Nashville, TN) RNAimage system for the differential display PCR. Each RNA sample is purified, DNAsed, and then reverse transcribed to cDNA in three reactions, each containing a different one base anchored poly T primer. Subsets of cDNAs are amplified using random upstream 10bp primers in combination with each of the 3 poly T primers, and each PCR reaction will be duplicated. The amplified cDNAs for each hormone treatment group (ICI, estrogen and tamoxifen) are run adjacently (including duplicates) on 6% polyacrylamide sequencing gels. The use of ^{33}PdCTP in the PCR reactions permits the autoradiographic detection of cDNAs. Differences in the expression of individual genes is observed visually, and can be quantitated with a phosphoimager. We anticipated that we would identify cDNAs that display varied patterns of hormonal regulation. We have been particularly interested in transcripts whose expression is induced by estrogen and tamoxifen, or by both compounds. However, we have also been interested in cDNAs that are downregulated by the ER ligands, as these may illustrate interesting mechanisms of transcriptional regulation in further studies. All differentially displayed bands are cut out of the gels, and the cDNA is extracted from the gel slices and used for PCR reamplification with the original primers. A total of 80 different upstream primers are available for differential display PCR, and the use of the complete set would enable us to examine the hormonal regulation of the total pool of genes expressed in MCF-7 cells. I have used 24 upstream primers in combination with the 3 poly T primers for my studies thus far.

3. Confirmation of the expression pattern of each mRNA.

Rationale and Methods

My PCR analyses resulted in the identification of a substantial group of cDNAs that appeared to be regulated by estrogen, tamoxifen, or by both compounds. However, I have been careful in selecting those to investigate further- this is necessary due to the high frequency of false positives which can be obtained using differential display PCR (26). In our study, these would refer to those cDNAs that appear to be regulated by estrogen or tamoxifen, but whose distinctions on differential display gels are the manifestations of PCR artifacts. Therefore, I have established a strict set of criteria for selecting those genes that are authentically regulated by estrogen and tamoxifen.

1. In aim #2, I have been duplicating all PCR reactions, and selecting only those differentially expressed cDNAs that are represented in identical quantities between duplicate PCR reactions. This was the basis for initially selecting our 13 candidate cDNAs.

2. The cDNAs selected in aim #2 must exhibit an identical expression pattern when differential display PCR is repeated with the same primer sets.

3. The cDNAs must show the same expression patterns as in the differential display when used as probes for northerns.

Genes that fulfill criteria 1 and 2 have been or will be sequenced and cloned. This will ensure that I achieve homogeneity in the purified PCR products prior to their further analysis. In addition, I have been using GENBANK to determine whether any of my candidates represent previously identified genes. Those cDNAs whose hormonal regulation is confirmed by northern analysis will be saved. Nuclear run on assays will then be performed with the cDNA(s) to investigate whether the ER effect is at a transcriptional level (which would validate cloning of the promoter). At this point I will have identified surrogate markers of estrogen and tamoxifen action in the MCF-7 breast cancer cell line. These genes will be the starting materials for further studies (as detailed below).

Preliminary data

I have analyzed most of the candidate ER regulated genes from the differential display, and unfortunately have found that most do not repeat their patterns of regulation that were observed by PCR, when they are analyzed by northern hybridization. One significant problem that we have encountered is contamination of our PCR reaction with unspliced RNAs and mitochondrial DNA. For these reasons, I have decided to use poly A RNA for any future differential display experiments.

My most promising result has been the identification of a cDNA that appears to be induced by both estrogen and the ER antagonist ICI in both MCF-7 and T47D breast cancer cells (Fig. 1, please see Appendices).

4. Cloning of the full length cDNAs and promoter regions.

Analysis of the regulation of newly identified targets of estrogen and tamoxifen action will necessitate cloning of the promoter regions. For those genes which have been previously cloned, the sequences will be obtained from the database and RT PCR will be used to obtain the full length cDNAs. Alternatively, the complete cDNAs of any novel genes will be identified by cDNA library screening. RACE (rapid amplification of cDNA ends) PCR (29) will be used to map the 5' ends of the cDNAs. The cDNAs will be used as probes for genomic library screening in order to identify the promoter regions of these genes. To ensure that I have cloned the promoter sequences, I will perform transient transfection assays in MCF-7 cells. The putative promoter regions of the genes will be

cloned into a luciferase reporter plasmid. This will be co-transfected with expression vectors for the human estrogen receptor (pRST7ER) and an RSV β-galactosidase plasmid to control for transfection efficiencies. The transfections will be followed by a 24 hour incubation in the presence or absence of 10^{-7}M estradiol or tamoxifen. Transcription will be quantitated by luciferase assays and B-galactosidase normalization procedures as described previously (9).

Results

I have screened an MCF-7 breast cancer cDNA library with our most promising ER-regulated transcript, and obtained the full length cDNA. I also used RACE PCR to map the 5' end of the cDNA. Although the identity of this gene was not known at the time of screening, it has since been identified (28) as the human mitochondrial intermediate peptidase (hMIP). Since I have performed experiments that show hMIP regulation by ER in the presence of cycloheximide, we have concluded that the cDNA is probably a direct target of ER. This initiated my interest in cloning the promoter, so that I could investigate the mechanism of ICI regulation of the gene. I have used the 5' end of the cDNA to screen a human genomic library and have obtained several genomic clones. I am currently using restriction digests and southerns to map and subclone the region immediately upstream of the 5'-terminus of the hMIP cDNA.

5. Characterization of the differential transcriptional activities of ERα and ERβ in response to agonists and antagonists

Due to recent observations that ERα and ERβ are both expressed in breast tumors, I was also interested in characterizing the relative abilities of the two ERs to regulate their known target promoters in the presence of different ER ligands. In order to compare the transcriptional activities of ERα and ERβ, we used the pRST7ER and pRST7ERβ expression vectors that encode the hERα and hERβ. We chose HepG2 (human hepatoma) and HeLa (human cervical carcinoma) cells for our studies, because they contain no endogenous ER and this enabled us to measure the transcriptional responses of each receptor in isolation. The ER expression vectors were transiently transfected into HepG2 and HeLa cells together with the estrogen-responsive reporter 3x-ERE-TATA-Luc or C3-Luc. We first examined the transcriptional responses of ERα and ERβ to the agonist estradiol in parallel dose-response studies (Fig. 2A, B). In the absence of hormone, ERα showed a significantly higher level of basal activity than ERβ in both HepG2 (Fig. 2A) and HeLa Cells (Fig. 2B), on both the simple and complex promoters. Addition of increasing concentrations of agonist resulted in a linear dose-dependent increase in the transcriptional activity of both receptors. However, estradiol had a significantly lower EC50 value on ERα when compared to ERβ in both HepG2 (Fig.2A) and HeLa cells (data not shown).

Furthermore, the efficacies displayed by ERα were consistently higher than those displayed by ERβ under the conditions we used. These results were also seen in transient transfection assays performed in other cell lines. Based on these studies we conclude that estradiol is a more potent and efficacious activator of ERα.

Antiestrogens can be divided into two broad categories based upon their abilities to differentially activate ER. The antiestrogen ICI acts as a pure antagonist in that it antagonizes ER function in all known cell and promoter contexts (11). Furthermore, ICI has been shown to decrease basal receptor activity, a phenomenon known as inverse agonism. The triphenylethylene compounds tamoxifen and GW 7604, and the benzothiophene-derived chemical raloxifene are mixed agonists, because they have been shown to manifest partial agonist activity through ERα in selected cell and promoter contexts (9), while acting as classical ER competitive antagonists in most target cells. Our previous studies have shown that antiestrogens also act as competitive antagonists of ERβ (unpublished data), however, the agonist functions of these compounds have not been extensively characterized with both ER subtypes. Therefore, we compared the ability of different antiestrogens to activate ERα and ERβ in dose-response studies. HepG2 and HeLa cells were transiently transfected with the ERα and ERβ expression vectors together with the C3-Luc reporter (Fig.2C) or 3x-ERE-TATA-Luc reporter (data not shown) and induced with increasing concentrations of different ER ligands. Treatment of both receptors with estradiol resulted in enhanced ERα activation compared to ERβ, as was observed in Figures 1A and B. Interestingly, the antiestrogens displayed no agonist or inverse agonist activities through ERβ on both promoters (Figure 2C and data not shown). However, tamoxifen displayed partial agonist activity through ERα in HepG2 cells on the C3-Luc reporter (Fig. 2C), and GW 7604 was a partial agonist on the 3x-ERE-TATA-Luc reporter in both HepG2 and HeLa cells (data not shown). Conversely, the antiestrogens ICI and raloxifene were inverse agonists of ERα in a dose-dependent manner in all contexts examined. Overall, these studies showed that ERα is significantly more responsive to both agonists and antagonists compared to ERβ. It will be interesting to repeat these studies using the novel genes that will be identified in this study.

6. Characterization of the expression and regulation of the novel estrogen and tamoxifen regulated genes by ERα and ERβ.

Methods

To complete my initial objectives, I will analyze the estrogen and tamoxifen regulation of the identified genes (from part 4) in MCF-7 cells by both ERα and ERβ. Transient transfection assays will be performed with mammalian expression vectors encoding hERα and hERβ, according to the following conditions:

(1) In the presence of increasing concentrations of estrogen or tamoxifen (and other antiestrogens) to study the kinetics of gene induction or downregulation.

(2) In the presence of different concentrations of both tamoxifen and estrogen for any genes shown to be regulated by both ligands. This will enable us to determine the relative ability of each ligand to regulate gene transcription.

(3) In different cell lines: transfections will be performed in Ishikawa cells (human endometrial carcinoma cells), where tamoxifen is an agonist for growth. Furthermore, we will examine the expression of all estrogen and tamoxifen regulated genes in the LY-2 breast cancer cell line, where the ER pathway is uncoupled from cell proliferation (this cell line has been used as a model for hormone resistant breast cancer). We will compare regulation of these genes in LY-2 cells to observations made in MCF-7 cells in order to observe differences in gene regulation associated with the hormone resistant state.

(4) In the presence of agents known to stimulate the ER pathway such as dopamine, growth factors (EGF, IGF, TGFα) and cAMP. The ability of estrogen and tamoxifen to potentiate this activity will be tested. These experiments will be repeated in LY-2 cells in order to examine the contributions of these factors to ER activity and gene regulation in the hormone resistant state.

(5) Tamoxifen regulated genes will be co-transfected with fos and jun in order to investigate whether their mechanism of regulation involves AP-1 as previously proposed.

Having completed these studies, we will define the promoter elements responsible for estrogen or tamoxifen regulation.

CONCLUSIONS

I have used differential display PCR to identify surrogate markers of estrogen and antiestrogen action in human breast cancer cells. One gene was chosen for further study due to its ability to be upregulated by both an estrogen receptor agonist and antagonist. I have since cloned the full length cDNA and identified the gene as hMIP (human mitochondrial intermediate peptidase), an enzyme important in cellular respiration. My current studies are aimed at characterizing the ER regulation of hMIP and defining the promoter sequences important for its regulation by agonist and antagonist. Since hMIP is known to be important in the processing of many enzymes that are essential for cell growth, it would be interesting to see whether this gene is upregulated in ER-positive breast tumors and in tamoxifen resistant tumors. Furthermore, it would be interesting to observe whether the levels of expression decrease as some breast tumors assume estrogen-independent growth. Finally, it will be important to determine what the physiological significance of hMIP regulation by both estrogen and the antiestrogen ICI is. The commonality between these two compounds is their steroid backbones, so I am currently investigating whether some of the nonsteroidal ER agonists and antagonists can regulate hMIP expression.

The cDNAs of other potentially estrogen receptor regulated genes have also been obtained in my studies, although I have not been able to confirm their regulation by northern analysis thus far. I plan to continue analyzing the rest of these cDNAs, and to continue with differential display PCR to look for additional ER regulated cDNAs.

I have also expanded my project to include an analysis of the differential transcriptional properties of the two estrogen receptor (ERα and ERβ). Since both receptors are expressed in breast tumors, it is important to understand how the two receptors differ in their regulation of gene expression. While both receptors are thought to regulate the same genes, the fact that ERβ is less sensitive to estrogens and antiestrogens may suggest that the relative levels of the two receptors in a breast tumor could determine responsiveness to antiestrogen therapy, and the sensitivity of the tumor to estrogen-stimulated growth. It will be interesting to study the kinetics of transcriptional regulation of novel ER regulated genes identified in this study by both ERα and ERβ in response to estrogen and antiestrogens..

REFERENCES

1. Love, R.R. and Koroltchouk, V. 1993. Tamoxifen therapy in breast cancer control worldwide. *Bull. World Health Organ.* **71**, 795-803.

2. Wittliff, J.L. 1984. Steroid-hormone receptors in breast cancer. *Cancer* **53**, 630-643.

3. Sunderland, M.C. and Osborne, C.K. 1991. Tamoxifen in pre-menopausal patients with metastatic breast cancer: a review. *J. Clin. Oncol.* **9**, 1283-1297.

4. Marshall, E. 1995. Reanalysis confirms results of tainted study. *Science* **270**, 1562.

5. Morrow, M. and Jordan, V.C. 1993. Molecular mechanisms of resistance to tamoxifen therapy in breast cancer. *Arch. Surg.* **128** , 1187-1191.

6. Horwitz, K.B. 1994. How do breast cancers become hormone resistant? *J. Steroid Biochem. Molec. Biol.* **49**, 295-302.

7. Evans, R.M. 1988. The steroid and thyroid receptor superfamily. *Science* **240**, 889-895.

8. Tsai, M. and O'Malley, B.W. 1994. Molecular machanisms of action of steroid/thyroid receptor superfamily members. *Annu. Rev. Biochem.* **63**, 451-486.

9. Tzuckerman, M.T., Esty, A., Santiso-Mere, D., Danielian, P., Parker, M.G., Stein, R.B., Pike, J.W. and McDonnell, D.P. 1994. Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. *Mol. Endocrinol.* **8**, 21-30.

10. Berry, M., Metzger, D. and Chambon, P. 1990. Role of the activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen. *EMBO* **9**, 2811-2818.

11. McDonnell, D.P., Clemm, D.L., Hermann, T., Goldman, M.E. and Pike, J.W. 1995. Analysis of estrogen receptor function *in vitro* reveals three distinct classes of antiestrogens. *Mol. Endocrinol.* **9**, 659-669.

12. Mosselman, S., Polman, J. and Dijkema, R. 1996. ERβ: identification and characterization of a novel human estrogen receptor. *FEBS Let.* **392**, 49-53.

13. Kuiper, G. and Gustafsson, J. 1997. The novel estrogen receptor-β subtype: potential role in the cell- and promoter-specific actions of estrogen and anti-estrogens. *FEBS Let.* **410**, 87-90.

14. Couse, J.J., Lindsey, J., Grandien, K., Gustafsson, J. and Korach, K. 1997. Tissue distribution and quantitive analysis of estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) messenger ribonucleic acid in the wild-type and ER-α knockout mouse. *Endocrinol.* **138**, 4613-4621.

15. Cowley, S.M., Hoarse, S., Mosselman, S. and Parker, M.G. 1997. Estrogen receptors α and β form heterodimers on DNA. *J. Biol. Chem.* **272**, 19858-19862.

16. Pettersson, K., Grandien, K., Kuiper, G. and J. Gustafsson. 1997. Mouse estrogen receptor β forms estrogen response element-binding heterodimers with estrogen receptor α. *Mol. Endocrinol.* **11**, 1486-1496.

17. Greene, G.L. 1997. "Estrogen receptor modulators and targets." Presentation from Frontiers in Estrogen Action Meeting held May 7-9, 1997.

18. Montano, M.M. and Katzenellenbogen, B.S. 1997. The quionone reductase gene: A unique estrogen receptor-regulated gene that is activated by antiestrogens. *Proc. Natl. Acad. Sci. USA* **94**, 2581-2586.

19. Silva, I.D., Salicioni, A.M., Russo, I.H., Higgy, N.A., Gebrim, L.H. and Russo, J. 1997. Tamoxifen down-regulates CD36 messenger RNA levels in normal and neoplastic human breast tissues. *Cancer Res.* **57**, 378-381.

20. Gottardis, M.M., Robinson, S.P. and Jordan, V.C. 1988. Estradiol-stimulated growth of MCF-7 tumors implanted in athymic nude mice: a model to study the umoristatic action of tamoxifen. *J. Steroid. Biochem.* **30**, 311-314.

21. Gottardis, M.M. and Jordan, V.C. 1988. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res.* **48**, 5183-5187.

22. Gottardis, M.M., Jiang, S., Jeng, M. and Jordan, V.C. 1989. Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. *Cancer Res.* **49**, 4090-4093.

23. O'Malley, B.W., Schrader, W.T., Mani, S., Smith, C., Weigel, N.L., Conneely and Clark, J.H. 1995. An alternate ligand-independent pathway for activation of steroid receptors. *Recent Prog Horm Res.* **50**, 333-347.

24. Brown, A., Jeltsch, J., Roberts, M. and Chambon, P. 1984. Activation of pS2 gene transcription is a primary response to estrogen in the human breast cancer cell line MCF-7. *Proc. Natl. Acad. Sci. USA* **81**, 6344-6348.

25. Liang, P. and Pardee, A.B. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**, 967-971.

26. Ayala, M., Balint, R.F., Fernandez-de-Cossio, M.E., Canaan-Haden, L., Larrick, J.W. and Gavilondo, J.V. 1995. New primer strategy improves precision of differential display. *Biotechniques* **18**, 844-850.

27. Frohman, M.A., Dush, M.K. and Martin, G.R. 1988. Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* **85**, 8998-9002.

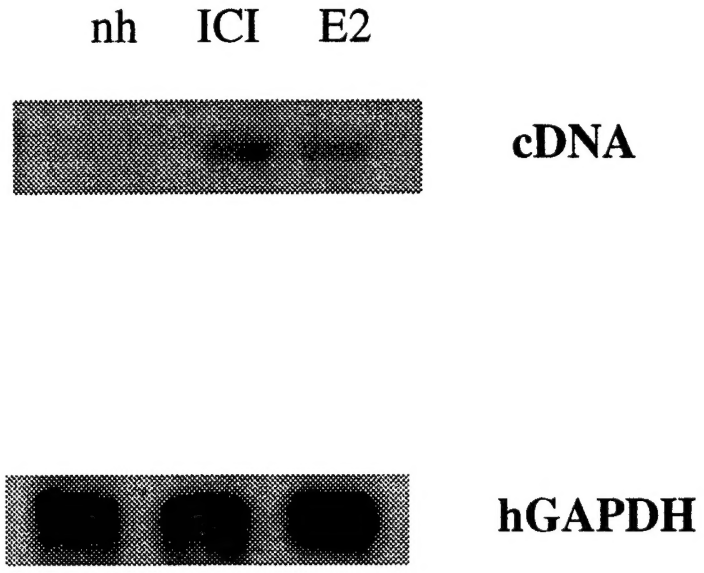
28. Chew, A., Buck, E.A., Peretz, S., Sirugo, G., Rinaldo, P. and Isaya, G. 1997. Cloning, expression, and chromosomal assignment of the human mitochondrial intermediate peptidase gene (MIPEP). *Genomics* **40**, 493-496.

APPENDICES

Figure 1

Identification of a cDNA induced by an ER agonist and antagonist in T47D breast cancer cells.

T47D breast cancer cells were treated for 6 hours with the ER agonist estrogen (E2), with the ER antagonist ICI or in the absence of hormone (nh). Total RNA was isolated, and run on a formamide gel and transferred to a nylon membrane. Northern blot analysis was performed using the 32-P labeled cDNA, which was identified by differential display PCR. A cDNA probe for the human glycerophophate dehydrogenase message (hGAPDH) was used for normalization.



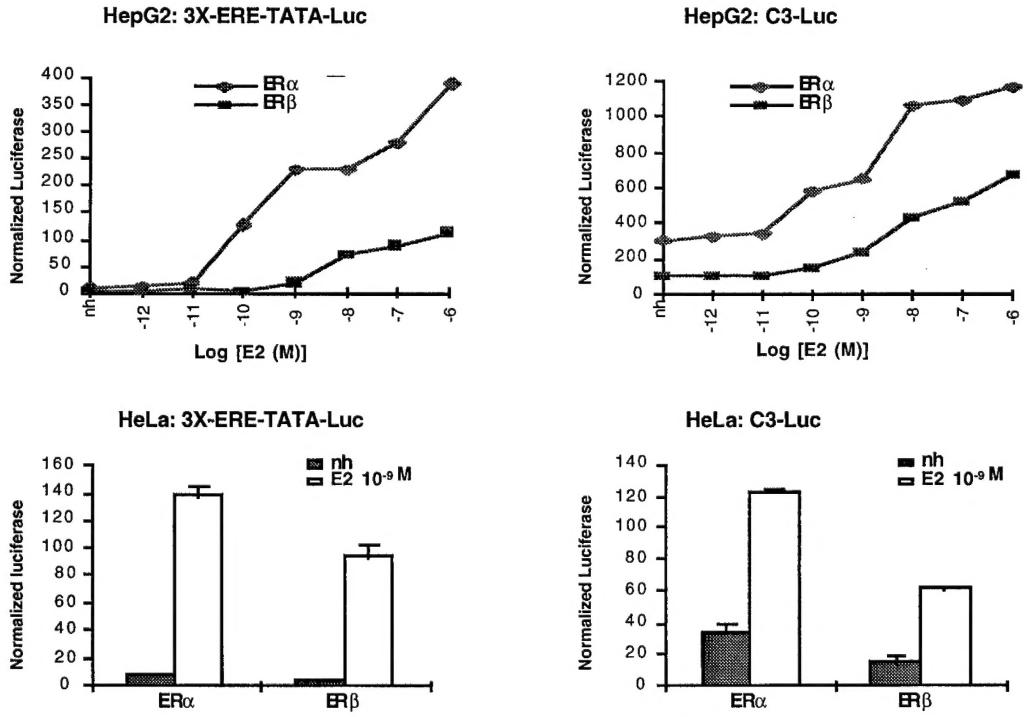
APPENDICES

Figure 2

Differential transcriptional activities of ERα and ERβ in response to estrogen and antiestrogens.

A. Dose response studies with estrogen. **B.** Dose response studies with estrogen and antiestrogens. HepG2 cells (A, B) and HeLa cells (A) were transiently transfected with ERα and ERβ expression plasmids, a constitutive β-galactosidase normalization plasmid, and 1 of 2 reporter vectors: 3X-ERE-TATA-Luc, containing 3 copies of an estrogen response element inserted upstream of an enhancerless luciferase reporter vector containing only a TATA element, or C3-Luc, which contains the complement 3 gene promoter fused upstream of the luciferase gene. Following a 48 hour hormone induction, transcription was quantitated by assaying for luciferase activity. All values were adjusted for transfection efficiency by normalization to β-galactosidase activity. Each data point is the average of a triplicate measurement, and the average coefficient of variation of each value is < 10%.

A.



APPENDICES

Figure 2

B.